Paired immunoglobulin-like receptor B (PIR-B) inhibits BCR-induced activation of Syk and Btk by SHP-1

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Coligation of paired immunoglobulin-like receptor B (PIR-B) with B cell antigen receptor (BCR) blocks antigen-induced B cell activation. This inhibition is mediated in part by recruitment of SHP-1 and SHP-2 to the phosphorylated ITIMs in the cytoplasmic domain of PIR-B; however the molecular target(s) of these phosphatases remain elusive. Here we show that PIR-B ligation inhibits the BCR-induced tyrosine phosphorylation of Ig α /Ig β , Syk, Btk and phospholipase C (PLC)- γ 2. Overexpression of a catalytically inactive form of SHP-1 prevents the PIR-B-mediated inhibition of tyrosine phosphorylation of Syk, Btk, and PLC- γ 2. Dephosphorylation of Syk and Btk mediated by SHP-1 leads to a decrease of their kinase activity, which in turn inhibits tyrosine phosphorylation of PLC- γ 2. Furthermore, we define a requirement for Lyn in mediating tyrosine phosphorylation of PIR-B. Based on these results, we propose a model of PIR-B-mediated inhibitory signaling in which coligation of PIR-B and BCR results in phosphorylation of ITIMs by Lyn, subsequent recruitment of SHP-1, and a resulting inhibition of the BCR-induced inositol 1,4,5-trisphosphate generation by dephosphorylation of Syk and Btk.

Keywords: PIR-B; ITIM; SHP-1; SHP-2; tyrosine phosphorylation

Introduction

Antigen-mediated clustering of B cell antigen receptor (BCR) induces a transient protein tyrosine kinase (PTK) activity that activates several biochemical pathways and culminates in proliferation, differentiation, or apoptosis. Three distinct types of PTKs have been found to be activated upon BCR engagement: Src-family PTKs (including Lyn, Fyn, and Blk), Syk, and Btk. A multistep model for BCR signaling has been proposed in which Lyn is the first to be activated after BCR stimulation and phosphorylates the tyrosine residues in the ITAM sequences (for immunoreceptor tyrosine-based activation motif) within Ig α and Ig β subunits. The phosphorylated ITAMs recruit Syk, which becomes phosphorylated and activated. Critical downstream signaling events include the activation of

phospholipase C (PLC)- γ 2, which is mediated by Syk and Btk. The activated PLC- γ 2 converts phosphatidylinositol 4,5-bisphosphate into the two second messengers diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP₃) which results in activation of protein kinase C and increases in [Ca²⁺]_i, respectively (for reviews see references: DeFranco, 1997; Kurosaki, 1997; Pleiman *et al.*, 1994; Reth and Wienands, 1997).

B cells express paired immunoglobulin-like receptor B (PIR-B), an integral membrane protein that contains six Ig-like domains in the extracellular portion and four potential ITIMs (for immunoreceptor tyrosine-based inhibitory motifs) in the cytoplasmic domain (Hayami *et al.*, 1997; Kubagawa *et al.*, 1997). We and others have recently demonstrated that PIR-B inhibits the BCR signaling in a phosphotyrosine-dependent manner and that, among four potential ITIMs, Tyr⁷⁷¹ and Tyr⁸⁰¹ are critical for mediating inhibitory signals (Figure 1) (Bléry *et al.*, 1998; Maeda *et al.*, 1998). Although the ligand for PIR-B is still unknown, PIR-B is inert until co-ligated to BCR at which time it undergoes tyrosine phosphorylation of ITIMs in its cytoplasmic domain. This phosphorylation leads to the recruitment of SHP-1 and SHP-2, which are responsible for the inhibition of calcium mobilization in the stimulated B cells.

In this report, we have examined the molecular mechanism underlying PIR-B-mediated inhibition of B cell activation. We first provide genetic evidence that Lyn is specifically required for tyrosine phosphorylation of critical ITIMs in the PIR-B cytoplasmic domain and its association with SHP-1 and SHP-2. The associated tyrosine phosphatases mediate dephosphorylation of Syk, Btk, and PLC- γ 2. Since activation of Syk and Btk is essential for PLC- γ 2 activation upon BCR engagement, our results suggest that down-regulation of Syk and Btk activity by SHP-1 and SHP-2 is a potential mechanism for inhibition of IP₃ generation and calcium mobilization by PIR-B.

Results

Recruitment of SHP-1 and SHP-2 to PIR-B ITIMs

Chimeric receptor Fc γ RIIB-PIR-B with the cytoplasmic domain of PIR-B and the extracellular domain of mouse Fc γ RIIB inhibits activation of B cells and mast cells like native PIR-B (Bléry *et al.*, 1998; Maeda *et al.*, 1998). Previous studies have demonstrated that among four potential ITIMs of PIR-B cytoplasmic domain (legends

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Figure 1 Tyr⁷⁷¹ and Tyr⁸⁰¹ in PIR-B are required for FcγRIIB/PIR-B-mediated inhibition of calcium mobilization and for recruitment of SHP-1 and SHP-2. (a) Schematic diagram of FcγRIIB/PIR-B chimeric molecules. PIR-B cytoplasmic region contains four potential ITIMs (residues 688–693, SLYASV; 717–722, ETYAQV; 769–774, VTYAQL; and 799–804, SVYATL). In addition to these tyrosines, the cytoplasmic domain of PIR-B contains one more tyrosine (residue 747). The open and shaded boxes represent portions of FcγRIIB and PIR-B, respectively. (b) Calcium mobilization stimulated by BCR crosslinking (solid line) and coligation of BCR to FcγRIIB/PIR-B (dashed line) in IIA1.6 cells expressing wild type and mutant FcγRIIB/PIR-B. Histogram in the inserted box indicates the expression level of FcγRIIB/PIR-B by 2.4G2 staining. Unstained cells were used as a negative control (open histogram). Stimulation of FcγRIIB/PIR-B alone by 2.4G2 did not induce calcium mobilization. (c) Immunoblotting with anti-SHP-1 (left panel) and anti-SHP-2 (right panel) Abs after BCR ligation alone and the coligation of BCR to mutant FcγRIIB/PIR-B. IIA1.6 cells expressing mutant FcγRIIB/PIR-B stimulated for 3 min with F(ab')₂ rabbit anti-mouse IgG (25 μg), or with intact rabbit anti-mouse IgG (50 μg) were lysed by NP-40 buffer. FcγRIIB/PIR-B was then immunoprecipitated with 2.4G2, resolved by SDS-PAGE gel, transferred to membrane, and probed with anti-SHP-1 Ab or anti-SHP-2 Ab. Membranes were reprobed with anti-PIR-B Ab (lower panel)

in Figure 1a), phosphorylation of both Tyr⁷⁷¹ and Tyr⁸⁰¹ is involved in PIR-B-mediated inhibitory signal presumably through recruitment of tyrosine phosphatases SHP-1 and SHP-2 (Bléry *et al.*, 1998; Maeda *et al.*, 1998). To formally demonstrate that the PIR-B cytoplasmic region containing doubly phosphorylated Tyr⁷⁷¹ and Tyr⁸⁰¹ is indeed the recruitment site for SHP-1 and SHP-2 *in vivo*, we transfected FcγRIIB-PIR-B mutant in which all remaining Tyr residues in the PIR-B cytoplasmic domain are changed to Phe (Y690, 719, 747F), into the FcγRIIB-negative B cell mutant cell line (A20 IIA1.6) (Figure 1a). Cell surface expression level of this chimeric receptor was checked by flow cytometric analysis using

anti-mouse FcγRIIB mAb, 2.4G2 (shown in Figure 1b inset box). As demonstrated in Figure 1b and c, coligation of FcγRIIB-PIR-B(Y690, 719, 747F) and BCR inhibited the calcium mobilization and recruited SHP-1 and SHP-2. Conversely, FcγRIIB-PIR-B(Y771, 810F) neither inhibited the BCR-induced calcium mobilization nor recruited SHP-1 and SHP-2. Three species of SHP-2 which migrated distinctly (right panel in Figure 1c) are presumably due to post-translational and/or post-transcriptional modifications of SHP-2, because three SHP-2 species similarly observed in DT40 B cells could not be detected after targeted deletion of the *SHP-2* gene (data not shown).

Lyn is required for tyrosine phosphorylation of PIR-B at Tyr⁷⁷¹ and Tyr⁸⁰¹

The above observations prompted us to examine the responsible PTK(s) for FcγRIIB-PIR-B(Y690, 719, 747F) tyrosine phosphorylation upon its coligation with BCR. For this purpose, we transfected FcγRIIB-PIR-B(Y690, 719, 747F) into *Lyn*- or *Syk*-deficient mutant DT40 cells (Takata *et al.*, 1994). After obtaining transformants expressing similar level of FcγRIIB-PIR-B(Y690, 719, 747F), DT40 cells deficient in *Lyn* or *Syk* were stimulated by BCR cross-linking alone or coligation of BCR and the inhibitory receptor (Figure 2). Consistent with our previous results using mouse A20 B cells (Maeda *et al.*, 1998), the chimeric receptor was slightly tyrosine phosphorylated upon BCR cross-linking alone and this phosphorylation was markedly augmented by coligation of BCR and FcγRIIB-PIR-B (Y690, 719, 747F) in wild type DT40 B cells. *Syk*-deficient DT40 cells showed the similar phosphorylation status of FcγRIIB-PIR-B(Y690, 719, 747F) to wild type cells, whereas this FcγRIIB-PIR-B(Y690, 719, 747F) phosphorylation was abrogated by loss of *Lyn*. These results demonstrate that *Lyn* is required for phosphorylation of Tyr⁷⁷¹ and Tyr⁸⁰¹ in the PIR-B cytoplasmic domain.

PIR-B inhibits PLC-γ2 activation

IP₃ generation by PLC-γ2 activation is essential for BCR-induced calcium mobilization from intracellular pools (Sugawara *et al.*, 1997; Takata *et al.*, 1995). Then, Ca²⁺ enters the cytoplasm by a process termed capacitative Ca²⁺ entry, by which the depletion of intracellular stores by IP₃ activates calcium influx across the plasma membrane (Putney and Bird, 1993). Thus, inhibition of the BCR-induced calcium mobilization by PIR-B could be accounted for by two possible mechanisms: inhibition of the BCR-induced IP₃ generation or inhibition of capacitative Ca²⁺ entry process. To distinguish these two possibilities, we determined IP₃ generation by BCR cross-linking alone or coligation of BCR and FcγRIIB-PIR-B. As shown in Figure 3a, BCR cross-linking alone generated IP₃ while this generation was abrogated by coligation of the inhibitory receptor and BCR. Consistent with this result, BCR-induced tyrosine phosphorylation of PLC-γ2 was profoundly inhibited by its coligation with FcγRIIB-PIR-B (Figure 3b). Thus, we conclude that coligation of BCR and FcγRIIB-PIR-B inhibits PLC-γ2 tyrosine phosphorylation, thereby leading to inhibition of IP₃ generation.

Figure 2 Tyrosine phosphorylation of Fc γ RIIB/PIR-B(Y690, 719, 747F) by BCR crosslinking alone and coligation in wildtype, Lyn-negative, and Syk-negative DT40 cells. The cells expressing Fc γ RIIB/PIR-B(Y690, 719, 747F) were incubated with F(ab')₂ rabbit anti-mouse IgM (10 μ g), or with intact rabbit anti-mouse IgM (20 μ g), then stimulated by anti-chicken IgM mAb M4 (4 μ g). Fc γ RIIB/PIR-B (Y690, 719, 747F) was immunoprecipitated with 2.4G2, separated by SDS-PAGE gel, transferred to membrane, and immunoblotted with anti-phosphotyrosine mAb 4G10 (upper panel). The same membrane was reprobed with anti-PIR-B Ab (lower panel)

Syk and Btk are potential targets of SHP-1

Early activation of PTKs, particularly Syk and Btk, is involved in PLC- γ 2 activation upon BCR engagement (Kurosaki, 1997). Therefore, data presented in Figure 3 could be explained by PIR-B-mediated dephosphorylation of PLC- γ 2 and/or its upstream PTKs, Syk and Btk. To examine these possibilities, the status of tyrosine phosphorylation of Syk and Btk was determined by immunoprecipitation followed by Western blotting with antiphosphotyrosine mAb. As shown in Figure 4a and b, the BCR-induced tyrosine phosphorylation of Syk and Btk was dramatically inhibited by coligation of BCR and Fc γ RIIB-PIR-B. Moreover, dephosphorylation of Syk and Btk by Fc γ RIIB-PIR-B resulted in inhibition of their kinase activity, as assessed by *in vitro* kinase assay. As reported previously (Burkhardt *et al.*, 1991; Yamanaishi *et al.*, 1992), Lyn is activated about 1.5-fold upon BCR stimulation alone (Figure 4c). In contrast to inhibition of Syk and Btk, coligation of the BCR and Fc γ RIIB-PIR-B did not inhibit the tyrosine phosphorylation and kinase activity of Lyn. Since binding of Syk to phosphorylated Ig α /Ig β is essential for participation of Syk in downstream signaling events such as PLC- γ 2 activation (DeFranco, 1997; Kurosaki, 1997; Pleiman *et al.*, 1994; Reth and Wienands, 1997), we also determined whether BCR-induced tyrosine phosphorylation of Ig α and Ig β is inhibited by its coligation with Fc γ RIIB-PIR-B. As shown in Figure 4d, coligation of the BCR and Fc γ RIIB-PIR-B inhibited phosphorylation of both Ig α and Ig β . Thus, these observations indicate that ligation of Fc γ RIIB-PIR-B inhibits BCR-induced tyrosine phosphorylation of four critical signaling molecules: Ig α /Ig β , Syk, Btk, and PLC- γ 2. Furthermore, dephosphorylation of Syk and Btk leads to inhibition of their kinase activity.

To investigate whether the Fc γ RIIB-PIR-B-mediated inhibition of tyrosine phosphorylation of signaling molecules could be attributed to SHP-1, we employed vaccinia virus-driven overexpression system of SHP-1 (Figure 5). Expression of the catalytically inactive

SHP-1-C453S, but not wild type SHP-1, significantly reversed the Fc γ RIIB-PIR-B-mediated inhibition of tyrosine phosphorylation of Syk, Btk, and PLC- γ 2 (Figure 6). Moreover, kinase activity of Syk and Btk was also restored by expression of SHP-1-C453S. In contrast, inhibition of Ig α tyrosine phosphorylation was less reversed than Syk and Btk. These results demonstrate that Fc γ RIIB-PIR-B ligation interrupts tyrosine phosphorylation of Syk, Btk, and PLC- γ 2 via an SHP-1 dependent mechanism.

Discussion

Recent studies have shown that among the five tyrosine residues in the PIR-B cytoplasmic domain, phosphorylation of Tyr⁷⁷¹ and Tyr⁸⁰¹ is required for its inhibitory mechanism (Bléry *et al.*, 1998; Maeda *et al.*, 1998). Here we extended these data to show that phosphorylation of remaining Tyr residues (Tyr⁶⁹⁰, Tyr⁷¹⁹, Tyr⁷⁴⁷) is dispensable for PIR-B-mediated inhibition and that SHP-1 and SHP-2 are recruited to the PIR-B Tyr⁷⁷¹ and Tyr⁸⁰¹ ITIMs *in vivo*. This observation, together with the evidence that both SHP-1 and SHP-2 are able to bind to the phosphorylated ITIM peptide including Tyr⁷⁷¹ or Tyr⁸⁰¹ *in vitro* (Bléry *et al.*, 1998), indicates that association of SHP-1 and SHP-2 to the PIR-B Tyr⁷⁷¹ and Tyr⁸⁰¹ ITIMs is mediated via an SH2-phosphotyrosine interaction.

Data presented here demonstrate that Lyn, but not Syk, is required for PIR-B ITIM tyrosine phosphorylation in DT40 B cells. Because Lyn is dominantly expressed among Src-family PTKs particularly in DT40 cells (Takata *et al.*, 1994), it is likely that other Src-family PTKs such as Fyn and Blk are also capable of phosphorylating PIR-B ITIMs. Since Lyn is apparently involved in activating BCR signal by phosphorylating ITAMs in Ig α /Ig β and Syk (DeFranco, 1997; Kurosaki, 1997; Pleiman *et al.*, 1994; Reth and Wienands, 1997), our results support the contention that Lyn has both positive and negative roles in B cell activation (DeFranco, 1997; Kurosaki, 1997). The

Figure 3 Fc γ RIIB/PIR-B-mediated inhibitory effect on PLC- γ 2. (a) Phosphoinositide hydrolysis after BCR crosslinking alone and coligation. IIA1.6 cells expressing wild type Fc γ RIIB/PIR-B were stimulated with F(ab')₂ rabbit anti-mouse IgG (10 μ g), or with intact rabbit anti-mouse IgG (20 μ g). Soluble inositol was extracted and separated by the AG 1-X8 ion exchange columns. (b) Tyrosine phosphorylation of PLC- γ 2. After stimulation, PLC- γ 2 was immunoprecipitated with anti-PLC- γ 2 serum, separated by SDS-PAGE gel, transferred to membrane, and immunoblotted with anti-phosphotyrosine mAb 4G10 (upper panel). The same membrane was reprobbed with anti-PLC- γ 2 serum (lower panel).

negative role of Lyn appears to be mediated in part by PIR-B as well as Fc γ RIIB and CD22 (Chan *et al.*, 1997; Nishizumi *et al.*, 1998; O'Rourke *et al.*, 1997; Wang *et al.*, 1996). Slight PIR-B tyrosine phosphorylation by BCR alone, although less efficient than occurring after coligation of BCR and PIR-B (Figure 2), might provide insights into the mechanism by which PIR-B modulates B cell activation *in vivo*. Assuming that a small fraction of SHP-1 or SHP-2 is recruited to the slightly phosphorylated PIR-B by BCR ligation alone, a small portion of the inhibitory function of PIR-B might not necessarily require coligation of BCR and PIR-B. Nevertheless, it is clear that coligation of BCR and PIR-B inhibits BCR activation more dramatically.

Here we explored the molecular mechanisms of PIR-B-mediated inhibition of B cell activation. In contrast to Fc γ RIIB inhibition (Gupta *et al.*, 1997; Hippen *et al.*, 1997; Nadler *et al.*, 1997; Ono *et al.*, 1997), BCR-induced activation of the upstream tyrosine kinases Syk and Btk, as well as many other substrates were drastically inhibited by its coligation with PIR-B, strengthening our previous conclusion that PIR-B inhibits the B cell activation by utilizing a different inhibitory mechanism than Fc γ RIIB. Thus, PIR-B apparently belongs to a class of KIR-type inhibitory receptors (Ono *et al.*, 1997). Indeed, similar to PIR-B-mediated inhibition, coligation of FcR and KIR inhibits tyrosine phosphorylation of many of substrate phosphorylated by stimulation of the FcR alone (Binstadt *et al.*, 1996).

Figure 4 Fc γ RIIB/PIR-B-mediated inhibition on Syk, Btk, and Lyn. Tyrosine phosphorylation of Syk, Btk, Lyn, and Ig α (a–d) and *in vitro* kinase activity of Syk (middle panel in a), Btk (middle panel in b), and Lyn (middle panel in c). After stimulation, IIA1.6 cells expressing wild type Fc γ RIIB/PIR-B were lysed and immunoprecipitated with anti-Syk (a), anti-Btk (b), anti-Lyn (c), or anti-Ig α (d). As shown previously (Kurosaki *et al.*, 1994), another slower migrating Syk species was observed upon BCR stimulation. Immunoprecipitates were divided, and half of them were used for Western blotting with mAb 4G10 (upper panel). After the filter was stripped, the same blot was reprobbed (lower panel). The remaining half were used for *in vitro* kinase assay (middle panel in a–c). Reaction samples in Syk were separated by 12.5% SDS-PAGE gel, transferred and immunoblotted with anti-phosphotyrosine mAb 4G10. For Btk and Lyn, samples were electrophoresed by 8% SDS-PAGE gel.

Since IP₃ generation and its binding to IP₃ receptors are essential for the BCR-induced calcium mobilization from intracellular pools (Sugawara *et al.*, 1997; Takata *et al.*, 1995), our data indicate that PIR-B aborts the initial release of Ca²⁺ from the intracellular pools by inhibiting IP₃ generation through down-regulation of PLC- γ 2. As SHP-1 and SHP-2 are required for PIR-B-

mediated inhibition, coligation of FcR and KIR inhibits tyrosine phosphorylation of many of substrate phosphorylated by stimulation of the FcR alone (Binstadt *et al.*, 1996).

Figure 5 Expression of wild type SHP-1 and SHP-1C453S. Whole cell extracts from cells infected with vector virus (V) or recombinant virus encoding wild type SHP-1 (wt) and SHP-1 C453S (CS), were subject to SDS-PAGE gel, followed by Western blot analysis with anti-SHP-1

mediated inhibition of calcium mobilization (Maeda *et al.*, 1998), the simplest interpretation for inhibition of PLC- γ 2 tyrosine phosphorylation is that PLC- γ 2 serves as a direct substrate of SHP-1 and SHP-2. Assuming that PLC- γ 2 is a sole substrate of SHP-1 and SHP-2 by PIR-B, one would predict that engagement of PIR-B with BCR dephosphorylates only PLC- γ 2 rather than many substrates. However, as shown in this study, not only PLC- γ 2 but also several signaling molecules (Figure 4) were dephosphorylated by coligation of PIR-B and BCR. Furthermore, analyses of phosphotyrosine-containing proteins of whole cellular lysates indicate the inhibition of tyrosine phosphorylation of many proteins by PIR-B (data not shown). Thus, our data favor a model in which the activation of upstream PTKs rather than PLC- γ 2 itself are down-modulated by PIR-B ligation.

Among BCR-activated PTKs, Syk and Btk are particularly important, since genetic studies indicate the requirement of Syk and Btk for BCR-induced tyrosine phosphorylation of PLC- γ 2 (Fluckiger *et al.*, 1998; Scharenberg *et al.*, 1998; Takata and Kurosaki, 1996; Takata *et al.*, 1994). Indeed, BCR-induced activation of Syk and Btk was profoundly inhibited by coligation of PIR-B and BCR, and this inhibition was substantially recovered by overexpression of catalytically inactive SHP-1-C453S. As PIR-B-mediated inhibition requires redundant function of SHP-1 and SHP-2 (Maeda *et al.*, 1998), overexpression of SHP-1C-453S would likely block the function of both SHP-1 and SHP-2 to a similar extent. PIR-B-mediated dephosphorylation of Ig α and Ig β could also account for down-modulation of Syk, because tyrosine phosphorylation of Syk is followed by its association with phosphorylated Ig α /Ig β (DeFranco, 1997; Kurosaki, 1997; Pleiman *et al.*, 1994; Reth and Wienands, 1997). In contrast to substantial restoration of Syk and Btk phosphorylation by SHP-1-C453S, less restoration of Ig α /Ig β phosphorylation suggests that PIR-B-

Figure 6 Restoration of Fc γ RIIB/PIR-B-mediated inhibitory effects by overexpression of catalytically inactive SHP-1. Tyrosine phosphorylation of Syk, Btk, PLC- γ 2, and Ig α (a–d) and *in vitro* kinase activity of Syk (middle panel in a) and Btk (middle panel in b). IIA1.6 cells expressing wild type Fc γ RIIB/PIR-B (5×10^6 per lane) were infected with recombinant vaccinia encoding either wild type SHP-1 or catalytically inactive SHP-1-C453S. After stimulation (1 min), cells were lysed and immunoprecipitated with anti-Syk (a), anti-Btk (b), anti-PLC- γ 2 (c) or anti-Ig α (d). Immunoprecipitates were immunoblotted with mAb 4G10 (upper panel). After the filter was stripped, the same blot was reprobed (lower panel). Immunoprecipitates with anti-Syk or anti-Btk were divided, and half of them were used for Western blotting. The remaining half were used for *in vitro* kinase assay (middle panel in a and b)

mediated Ig α /Ig β dephosphorylation is mediated by another type of tyrosine phosphatase(s). Involvement of this phosphatase, in addition to SHP-1 and SHP-2, might explain the residual PIR-B inhibition in SHP-1 and SHP-2 double-deficient DT40 cells (Maeda *et al.*, 1998). In contrast to Syk and Btk, Lyn activation was not significantly inhibited by PIR-B ligation. Thus, our data indicate that among the three types of BCR-activated PTKs, Syk and Btk are specifically down-modulated by SHP-1 and SHP-2, thereby leading to inhibition of PLC- γ 2 phosphorylation.

In summary, our data support the following mechanism: (a) Coligation of PIR-B and BCR results in phosphorylation of PIR-B ITIMs by Lyn; (b) SHP-1 and SHP-2 are recruited to the phosphorylated PIR-B ITIMs via their SH2 domains, and are subsequently

activated; (c) activated SHP-1/SHP-2 dephosphorylate their substrates apparently including Syk and Btk; (d) down-modulation of Syk and Btk by presumably dephosphorylation of tyrosine residues in their activation loop inhibits PLC- γ 2 activation; (e) down-modulated PLC- γ 2 decreases IP₃ generation, leading to a block of calcium mobilization.

Materials and methods

Cells, expression construct and Abs

Various mutant DT40 cells and A20 IIA1.6 cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin, and glutamine. For making Fc γ RIIB/PIR-B chimera, the cDNA of mouse Fc γ RIIB (corresponding to amino acid residue: -29 to 207 including signal peptide) and PIR-B (corresponding to amino acid residue: 641-818) were fused by using PCR. Mutations that tyrosine residues at positions 690, 719, 747, 771 and 801 to phenylalanine were introduced by the PCR method. Resulting constructs were confirmed by DNA sequencing. The mutant and wild type Fc γ RIIB/PIR-B cDNAs were subcloned into pAuro vector (Takata *et al.*, 1994) and were electroporated into DT40 or A20 IIA1.6 cells as described previously (Sugawara *et al.*, 1997). After selecting clones in the presence of puromycin (0.5 μ g/ml), cell surface expression levels of Fc γ RIIB/PIR-B were checked by flow cytometry analysis using anti-mouse Fc γ RIIB mAb, 2.4G2 (Unkeless, 1979). Anti-chicken IgM mAb M4, anti-Syk Ab, anti-PLC- γ 2 Ab, anti-SHP-1 Ab, anti-Ig α Ab were already described (Maeda *et al.*, 1998; Sanchez *et al.*, 1993; Takata *et al.*, 1994). Anti-PIR-B Ab was obtained by immunizing rabbits with bacterially expressed glutathione S-transferase fusion protein containing PIR-B cytoplasmic region. Anti-SHP-2 Ab, intact rabbit anti-mouse IgM, F(ab')₂ rabbit anti-mouse IgM, intact rabbit anti-mouse IgG, F(ab')₂ rabbit anti-mouse IgG, and anti-phosphotyrosine mAb 4G10 were purchased from Santa Cruz Biotechnology Inc, Zymed laboratories Inc, Chemicon Inc, Chemicon Inc, and Upstate Biotechnology Inc, respectively. The anti-Btk mAb 43-3B was used for immunoprecipitation and Western blotting of Btk (Baba *et al.*, in press). Polyclonal anti-Lyn Ab used to immunoprecipitate was kindly provided by Drs H Umemori and T Yamamoto (Institute of Medical Science, University of Tokyo., unpublished data). Western blotting of Lyn was accomplished using the monoclonal anti-Lyn, Lyn 8 (Yamanashi *et al.*, 1992).

Vaccinia viruses

Recombinant vaccinia viruses encoding wild type and dominant-negative forms (cysteine 453 to serine) of SHP-1 have been described (Burshtyn *et al.*, 1996). For infections, A20 IIA1.6 cells (2 $\times$ 10⁶ cells/ml) were incubated in serum-free RPMI 1640 for 1 h at 37°C at a multiplicity of infection of 20. Cells were then incubated for 3 h at 10⁶ cells/ml in RPMI 1640 supplemented with 10% FCS.

Immunoprecipitation and Western blotting analysis

Cells were solubilized in lysis buffer (1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.5, 1 mM EDTA, 10% glycerol) containing 50 mM NaF, 10 μ M molybdate, 2 mM sodium vanadate supplemented with protease inhibitors as described previously (Sugawara *et al.*, 1997). For immunoprecipitation, precleared lysates were sequentially incubated with Abs and protein A-agarose or anti-rat IgG-

agarose. Lysates or immunoprecipitates were separated by SDS-PAGE gel, transferred to nitrocellulose membrane, and detected by appropriate Abs and ECL system (Amersham Corp.). To examine *in vitro* kinase activity of Syk, immunoprecipitated Syk was suspended in kinase buffer (20 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM MnCl₂) in the presence of ATP (1 μ M). Recombinant glutathione S-transferase fusion protein containing a cytoplasmic domain of mouse Ig α (5 μ g) was used as an exogenous substrate (Saouaf *et al.*, 1994). The reactions were allowed at 30°C for 10 min and terminated by the addition of sample buffer. The mixture was separated by SDS-PAGE gel, transferred to nitrocellulose membrane, and detected by 4G10 and ECL system. For *in vitro* kinase assay of Lyn and Btk, immunoprecipitates were suspended in kinase buffer (20 mM PIPES, pH 7.5, 20 mM MnCl₂) containing [γ -³²P]ATP (> 3000 Ci/mmol, Amersham), 5 μ g of enolase (Sigma) and incubated at 25°C for 3 min. The reaction was stopped by addition of sample buffer. The mixture was separated by SDS-PAGE gel, followed by autoradiography.

Calcium and phosphoinositide analysis

For calcium analysis, cells (5 $\times$ 10⁶) were suspended in PBS containing 20 mM HEPES (pH 7.2), 5 mM glucose, 0.025% BSA and 1 mM CaCl₂, and loaded with 3 μ M Fura-2/AM at 37°C for 45 min. Cells were washed twice, and adjusted to 10⁶ cells/ml. Continuous monitoring of fluorescence from the cell suspension was performed using Hitachi F-2000 fluorescence spectrophotometer (Hitachi Limited, Tokyo, Japan) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. Calibration and calculation of calcium levels were done as described (Grynkiewicz *et al.*, 1985). For phosphoinositide analysis, cells (10⁶/ml) were labeled with myo-³H-inositol (10 μ Ci/ml, 105 Ci/mmol, Amersham) for 5-6 h in inositol-free RPMI-1640 supplemented with 10% dialyzed FCS, then stimulated with intact or F(ab')₂ fragment of rabbit anti-mouse IgG. The soluble inositol phosphates were extracted with trichloroacetic acid at indicated time points, and applied to AG 1-X8 (formate form) ion exchange columns (Bio-Rad Laboratories) pre-equilibrated with 0.1 M formic acid. The columns were washed with 10 ml water and 10 ml of 60 mM ammonium formate/5 mM sodium tetraborate. Elution was performed with increasing concentrations of ammonium formate (0.1-0.7 M) (Berridge *et al.*, 1983).

Flow cytometric analysis for surface expression of Fc γ RIIB/PIR-B

Cells were washed, stained with FITC-conjugated 2.4G2 (PharMingen) and analysed by FACScan (Becton-Dickinson & Co.). The *x* axes and *y* axes for the histograms in inserted boxes (Figure 1b) indicate fluorescence intensity (4-decade-log scales) and relative cell number, respectively.

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